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13. SUPPLEMENTARY NOTES

14. ABSTRACT
BRCA1 is a tumor suppressor gene for hereditary breast and ovarian cancers.In collaborating with various binding partners, BRCA1 protein participates in multiplecellular functions. Characterization of these binding proteins of BRCA1 is therefore key tothe complete understanding of BRCA1's role in tumor suppression. Cofactor of BRCA1 (COBRA1)is a novel BRCA1-interacting protein identified in our laboratory. Subsequent work suggeststhat COBRA1 and BRCA1 share several functional commonalities in regulating high-orderchromatin structure and estrogen-dependent gene expression. During the past year, we madesignificant progress in elucidating the role of COBRA1 in breast cancers. By analyzingCOBRA1 expression in breast cancer clinical samples, we found that COBRA1 level was inverselycorrelated with breast cancer progression. Concordantly, depletion of COBRA1 in breastcancer cells led to elevated cell growth under suboptimal concentrations of estrogen both invivo and in vitro. Thus, our work uncovered an important role of COBRA1 in breast cancer.It also provides a solid and logical foundation for exploring a synergistic relationshipbetween BRCA1 and COBRA1 in the next phase of the funded study.

15. SUBJECT TERMS
BRCA1, tumor suppression, transcriptional regulation

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INTRODUCTION

Breast Cancer 1 (*BRCA1*) is a tumor suppressor gene, whose mutations predispose women to high risk of breast and ovarian cancers (1). The BRCA1 protein interacts with numerous protein partners and is implicated in various cellular functions, among which the DNA damage responses and transcriptional regulation are the two most extensively studied biological activities of BRCA1 (2). While functional characterization of BRCA1 in these two directions is historically carried out independently, both processes are likely to take place in the same cellular context. However, it is not clear whether direct crosstalk exits between these two BRCA1-mediated processes. Furthermore, it is unknown as to the potential mechanisms used by BRCA1 to coordinate these events.

To address these broad questions, we have proposed to study the role of BRCA1 in connecting transcription and DNA damage responses at gene loci where transcription is regulated by BRCA1. In particular, we were interested in examining (1) how BRCA1 would modulate transcription of its target genes in the presence of DNA damage, and (2) whether BRCA1 could contribute to the transition between transcription and DNA repair machineries at these loci before and after DNA damage. As the first step of tackling these problems, we have carried out a gene expression profiling study to search for BRCA1 transcriptional targets in the whole-genome scale. After statistical analysis and independent validation, a group of cancer-related genes was indeed revealed as the transcriptional targets of BRCA1, which could therefore served as the foundation for aforementioned mechanistic studies.

It is abundantly clear that BRCA1 does not act alone in tumor suppression. Mammary gland-specific *Brca1* knockout mice display relatively a low incidence (25%) and late onset of tumor development (11 months of age), which is in sharp contradictory to the high prevalence of breast cancers in *BRCA1* mutation carriers. This suggests that genetic and/or environmental modifiers may play roles in influencing *BRCA1*-associated tumorigenesis. To identify additional factors that may cooperate with BRCA1 in tumor suppression and gene regulation, our laboratory has identified cofactor of BRCA1 (COBRA1) as a BRCA1-interacting protein through a yeast two-hybrid screening (3). At the molecular level, COBRA1 and BRCA1 share several functional similarities. First of all, both proteins are preferentially expressed in luminal epithelial cells (4). Secondly, COBRA1 and BRCA1 can both induce large-scale chromatin reorganization (3). Thirdly, these two proteins have been demonstrated to interact with estrogen receptor α (ERα) and served as corepressors in modulating estrogen-dependent gene expression (4, 5). Last but not least, our work from the first year of this funding period has identified a large number of genes that are co-regulated by COBRA1 and BRCA1 through an unbiased screening with the microarray technology. Importantly, many of those genes, such as S100P, TIMP-1, and GABBR1, have been previously implicated in various types of cancers. Taken together, these findings suggest that COBRA1 and BRCA1 may indeed cooperate with each other to modulate gene expression. In addition, these discoveries also provide compelling rationales for further exploring the functional connection of BRCA1 and COBRA1 in tumorigenesis of the breast tissue.

In light of the physical and functional interactions between BRCA1 and COBRA1, we have decided to combine the studies of BRCA1 and COBRA1 in gene regulation and in breast cancer. As demonstrated by work from numerous laboratories, BRCA1's biological activity in various cellular functions is largely dictated by its interacting partners. Hence elucidating the exact molecular mechanism of COBRA1 and the functional significance of its interaction with BRCA1 could provide new insights to the role of BRCA1 in breast cancer. As illustrated in greater details in the following sections, in the past year of the funded research, we have examined COBRA1 expression in clinical samples from both normal and tumor tissues, and have also carried out a xenograft study to discriminate a causal or bystander effect of COBRA1 in breast cancer progression. Our findings uncover many exciting characteristics of COBRA1 in breast cancer development that share intriguing commonality with BRCA1. Thus, these exciting results provide a solid and logical foundation for exploring a synergistic relationship between BRCA1 and COBRA1 in the next phase of the funded study.

BODY

I. COBRA1 expression in cancer cell lines and clinical samples

As the first step of dissecting COBRA1's role in breast cancer, we checked COBRA1 expression in a panel of cancer cell lines as well as clinical tissue samples. Western blot analysis was used to analyze expression of COBRA1 in breast (MCF10A, MCF-7, SKBR3) and ovarian (ES2, SKOV3, H118) cancer cell lines with a polyclonal antibody that could specifically recognized the endogenous COBRA1 protein. Interestingly, COBRA1 was expressed at comparable level in all the cell lines except for SKBR3, which is an ERα-negative breast cancer cell line with aggressive growth and metastatic phenotype (Fig. 1A). Next, we used clinical samples to evaluate COBRA1 expression in both normal and tumor tissues. We first performed immunohistochemistry (IHC) on a normal tissue array that contains most of the tissue and cell types of the human body. Consistent with the cell line study, COBRA1 was universally expressed in most tissues. The epithelium layer of many organs displayed stronger expression of COBRA1 than cells in the stroma. The intensity and prevalence of COBRA1 expression in epithelium layer was organ-specific with those of the gastrointestinal tract, such as small intestine, and colon expressing highest level of COBRA1 in virtually 100% of their epithelial cells, whereas only a potion of the same type of cells in prostate and seminal vesicle were positive for COBRA1 staining. In cells that did express COBRA1, the signal was enriched in the nucleus, which was in line with its known function in the regulation of gene expression (Fig. 1B). We then used IHC to compare COBRA1 level in both normal and tumor samples. As shown in figure 1C, normal mammary epithelial cells showed a strong nuclear staining of COBRA1. In contrast, COBRA1 signal was markedly reduced in breast cancer tissues when compared with their normal counterparts (Fig. 1C).

Prompted by the finding of low expression of COBRA1 in advanced breast cancer cell line and breast cancer tissues, we examined COBRA1 expression in a cohort
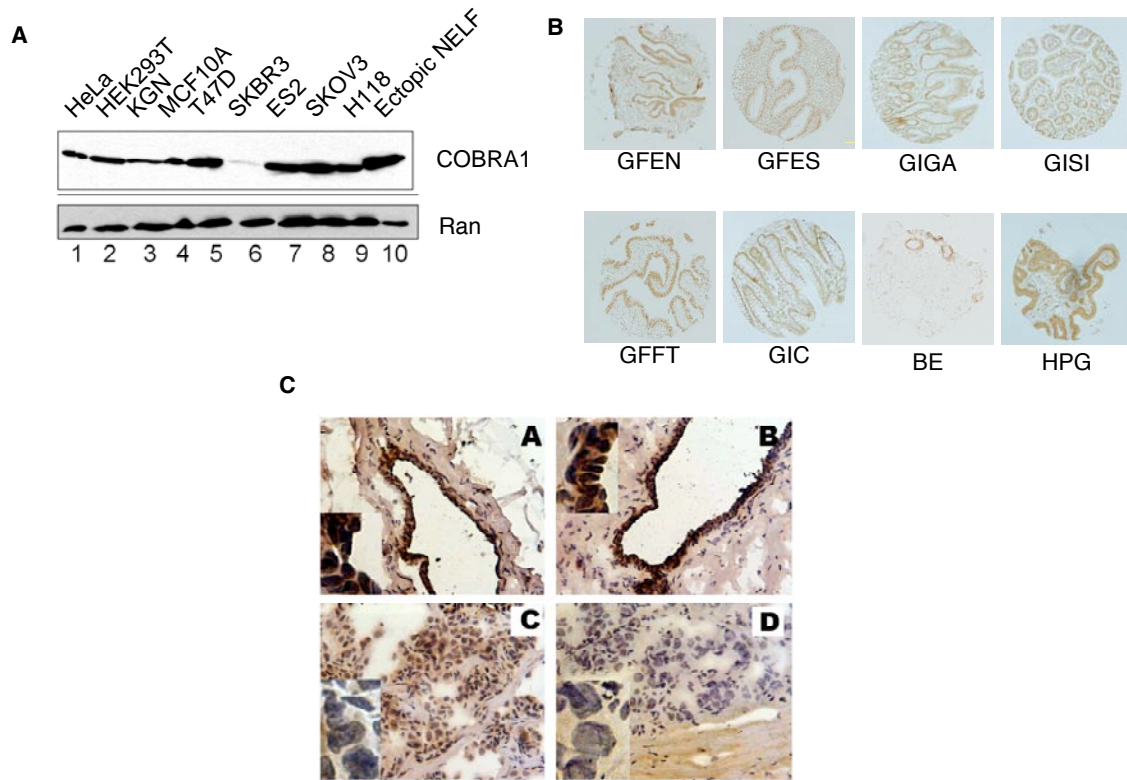
Figure 1. COBRA1 expression in established cell lines and clinical tissues. A. Western blot analysis of COBRA1 expression in multiple cell lines. The blot was probed anti-COBRA1 antibody. Ran was used as a loading control. **B.** Normal tissue array was immunostained with a COBRA1-specific polyclonal antibody. Several representative images of selective tissues are shown on the right. The images were taken at 100x magnification. Abbreviations are as following: GFEN, endocervix; GFES, endometrium, secretory; GIGA, gastric mucosa, antral; GISI, small intestine, mucosa; GFFT, fallopian tube; GIC, colon, mucosa; BE, breast epithelium; and HPG, gallbladder. **C.** Representative staining pattern of COBRA in normal mammary (panels A and B) and in tumor tissue (panels C and D). Mammary epithelial cells showed a strong nuclear pattern of staining of COBRA, whereas breast cancer cells showed a markedly reduced staining compared with normal epithelial cells. The panels are shown with X100 magnification, and the inserts with x400 magnifications.

of primary ductal carcinoma samples (n=87) collected immediately after mastectomy from patients who have been followed clinically for an average of 120 months since the surgery. Dependents on the clinical outcomes, the tumor samples were divided into four groups: (1) disease-free, (2) metastasis, (3) local recurrence, and (4) death of breast cancer (those who died of conditions unrelated to breast cancer were excluded in the analysis). As shown in Fig. 2A and 2B, tumors with metastasis and local recurrence had significantly lower levels of COBRA1 mRNA expression (p=0.0065 and 0.0081, respectively). Those tumors from patients who died eventually due to breast cancer also displayed marginally lower level of COBRA1 than disease-free group. Kaplan-Meier survival analysis revealed an interesting trend that low levels of COBRA1 transcript were associated with a shorter survival (116 (96-137, 95% CI) months) compared with high levels of COBRA1 (128 (115-142, 95% CI) months) (Fig. 2C). This difference

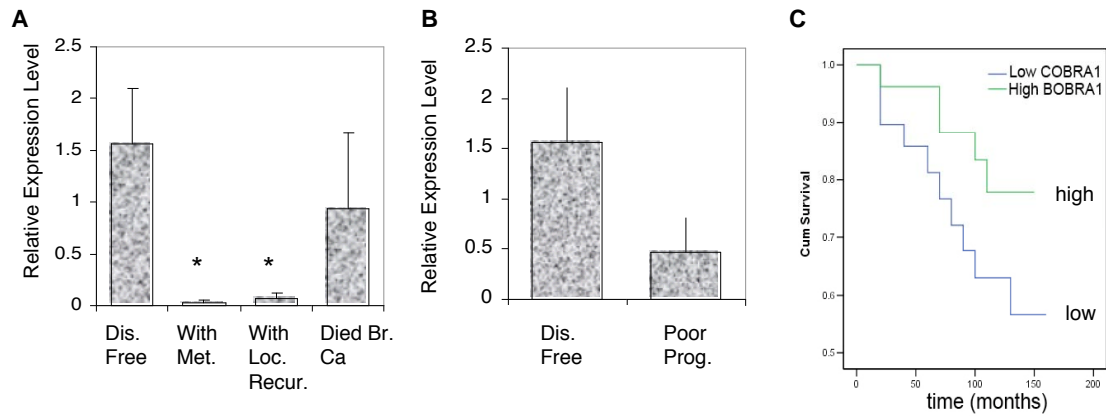nonetheless is not statistically significant (p=0.101), perhaps due to the relatively small size of the study cohort.

Figure 2. Quantitative RT-PCR analysis of COBRA1 mRNA expression in human ductal carcinoma tissues. **A.** Relationship between COBRA1 expression and clinical outcome over a 10-year follow-up period. There is a strong correlation between low COBRA1 levels and patients with metastasis or local recurrence (*p=0.0065 and 0.0081, respectively). **B.** Comparison between those patients who remained disease free and those who developed further disease progression (metastasis, recurrence and motality combined). **C.** Kaplan-Meier survival curve for a disease free survival.

II. Effect of COBRA1 depletion on tumor growth in xenograft model

The association of low COBRA1 expression with poor prognosis suggests that COBRA1 may be involved in various aspects of tumorigenesis of breast tissue. To ascertain such a causal effect of COBRA1, we tested the impact of COBRA1 depletion on tumor growth of breast cancer cell line ZR-75-1 in a xenograft model. The stable knockdown of COBRA1 protein in ZR-75-1 cells was achieved with a retrovirus-based shRNA expression system followed by neomycine selection (Fig. 3A). As control, virus that carried shRNA for luciferase was used for parallel infection. When seeded to the mammary fad pad of athymic mice in the presence of embedded estrogen pellet, both control and COBRA1 knockdown cells readily formed large tumors within a short period of time (< 4 weeks), and no significant difference in growth rate was observed between tumors derived from the pair of cells (Fig. 3B). In the absence of exogenously supplemented estrogen, however, tumors derived from both cell types during the same period were much smaller, which may reflect the strict estrogen-dependency of ZR-75-1 cells in proliferation (Fig. 3B). Interestingly, tumors from the COBRA1-depleted cells, but not the control cells, grew continuously after the initial tumor formation, and resulted in significantly lager tumor mass after growing for two more months (Fig. 3C).

We recovered the ZR-75-1 cells from the tumors and found the COBRA1 protein level remained low in the knockdown cells, which indicated that the stable reduction of
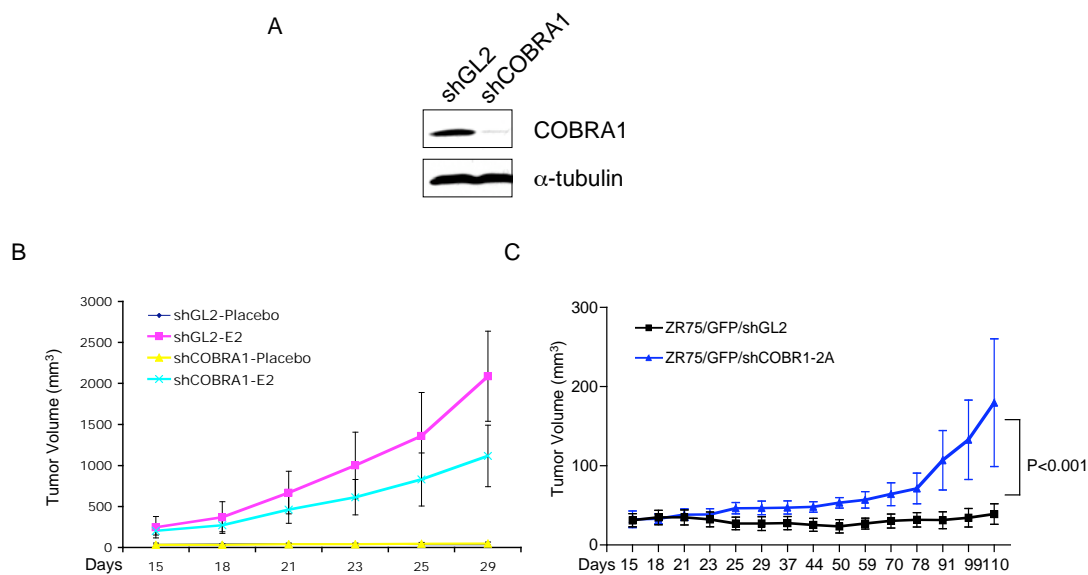
Figure 2. COBRA1 depletion led to continuous growth of ZR-75-1 cells in xenograft model under the low estrogen condition. **A.** Western blot analysis of COBRA1 level in control and shCOBRA1 expressing ZR-75-1 cells. α-tubulin was measued as loading control. **B.** Growth curve of tumors drived from cotrol and COBRA1 knockdown ZR-75-1 cells. Cells were inoculated in inguinal mammary gland in the presence of embedded estrogen or placebo pellets. Tumor volume was measued two weaks after cell injection and monitored every two to three days from then on. **C.** Tumor growth of control and COBRA1 knockdown cells in the absence of exogenousely supplemented estrogen pellet. The difference of tumor size between control and COBRA1 knockdown cells was statisticaly significant (p,0.001, F test).

COBRA1 was maintained during the in vivo process of tumor growth (Fig. 4A). As revealed by IHC, the COBRA1 knockdown tumors had a higher proportion of Ki67-positive cells (Fig. 4B), yet the percentage of apoptotic cells, as demonstrated by Caspase 3 activation, was comparable between the two types of tumors (Fig. 4C). Therefore, elevated proliferation was likely the cause for the observed difference in tumor growth. To measure the proliferation potential of control and COBRA1 knockdown cells in vitro, we performed BrdU incorporation assay at various concentrations of estrogen. Intriguingly, COBRA1 depletion led to an increased sensitivity to the suboptimal concentrations of estrogen in DNA synthesis, but no additional advantage was conveyed by COBRA1 knockdown when cells were supplied with optimal concentration of estrogen (Fig. 4D). Thus, this in vitro data, together with the in vivo observation, support the notion that reduction of COBRA1 facilitates cell proliferation in the presence of limited amount of estrogen for the ERα positive breast cancer cells.

While continuing on the COBRA1 front, we will also combine BRCA1 and COBRA1 in similar studies by using clinical samples and xenograft models. Specifically, it will be of great interest to address whether expression of BRCA1 and COBRA1 is co-regulated in breast cancer samples, and if so, what will be the clinical outcome when both proteins are co-reduced in primary tumors. In addition, we will test the effect of BRCA1 depletion on tumor growth of breast cancer cells by using the same xenograft model. The effect of co-depletion of both proteins on tumor growth will also
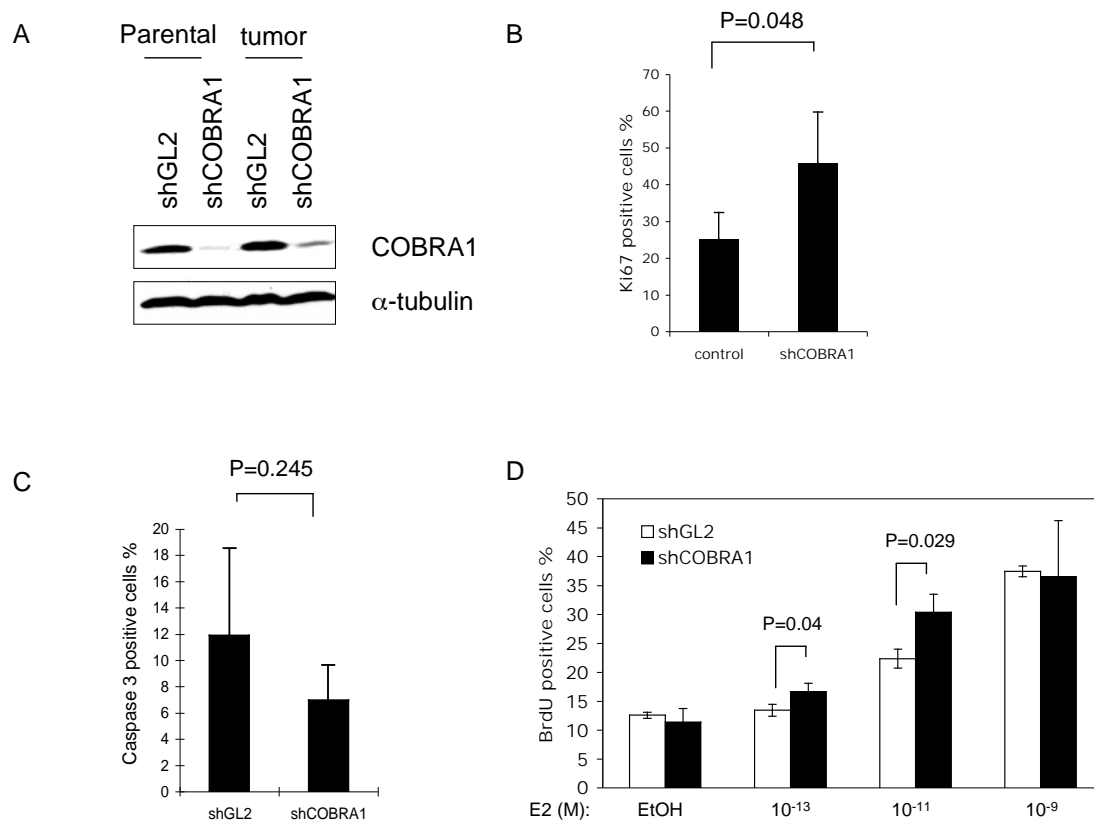
Figure 2. COBRA1 knockdown led to increased proliferation in suboptimal concentration of estrogen. A. Western blot analysis of COBRA1 level in control and COBRA1 knockdown ZR-75-1 cells obtained from tumors grown in the nude mice. α-tubulin was measued as loading control. **B.** Ki67 epxression in tumors from control and COBRA1 knockdown cells. Sections from fixed tumors were stained with Ki67 by IHC. Pecentage of Ki67 positive cells was determined from five randomly picked fields. Average was calculated from 4 tumors for both control and COBRA1 knockdown cells. **C.** Caspase 3 expression in tumors from control and COBRA1 knockdown cells was obtained in the same manner as Fig. 2B. **D.** BrdU incorporation assay for control and COBRA1 knockdown cells in vitro. Cells were starved in E2-free DMEM medium supplemented with 5% charcoal-stripped fetal bovine serum for three days. Estrogen was then added at various concentrations and maintained for 24 hours. Cells were pulse-labeled with 50μM of BrdU for the last four-hour of estrogen treatment, followed by immunocytochemistry analysis with Alex-488 conjugated anti-BrdU antibody. Percentage of BrdU positvie cells was caculated and average from three randomly selected field for each condition. Statistical analysis was performed by student's t test.

be examined, which will allow us to determine whether the collaboration between COBRA1 and BRCA1 in regulating gene expression will be translated into a functional synergy in suppressing tumor progression.

KEY RESEARCH ACCOMPLISHMENTS

- Examination of COBRA1 expression in clinical samples from both normal and tumor breast tissues.
- Xenograft study of COBRA1's role in modulating tumor growth of breast cancer cells.

REPORTABLE OUTCOMES

Sun J, Watkins G, Blair AL, Moskaluk C, Ghosh S, Jiang WG, Li R. Deregulation of cofactor of BRCA1 expression in breast cancer cells. J Cell Biochem. 2007 In press

CONCLUSION

The preliminary findings obtained during the second year of the current funding period made a significant contribution towards the understanding of COBRA1's role in breast cancer by demonstrating the reverse association of COBRA1 with disease progression and the causal relationship of COBRA1 reduction to tumor growth in vivo. These discoveries served as an important extension to our previous finding that COBRA1 and BRCA1 collaborate with each other to regulate gene expression in elucidating the functional involvement of COBRA1 in breast cancer. In the last year of the contract, we will continue to investigate the molecular mechanisms as to COBRA1's function in mammary tumorigenesis. Furthermore, it will be of great interest to explore the functional cooperation of COBRA1 and BRCA1 in suppressing tumor initiation and progression in breast cancers.

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